REMARKS/ARGUMENTS

Claims 30-70 are pending.

Applicant notes with appreciation the indicated allowability of claims 65-67 and 70.

Claims 30-64, 68 and 69 were provisionally rejected under the judicially created doctrine of obviousness-type double patenting as being unpatentable over claims 1-38 of U.S. Patent No. 6,632,658. Accordingly, applicant submits herewith a terminal disclaimer.

Claims 30-32, 36, 38-41, 49, 57, 60, 64 and 68 were rejected under 35 USC §102(b) as being anticipated by Starkie et al (WO 86-00636).

Claims 30, 31, 34-36, 38-43, 45, 50, 51, 56-59, 62-64, 68 and 69 were rejected under 35 USC §102(b) as being anticipated by Dean, Jr. et al. (U.S. Patent No. 4,978,616).

Claims 52-55 were rejected under 35 USC §103(a) as being unpatentable over Dean, Jr. et al. in view of Freedman et al. (U.S. Patent No. 5,501,971) and Schoeb et al. (U.S. Patent No. 6,100,618).

Claims 33, 37 and 61 were rejected under 35 USC §103(a) as being unpatentable Dean, Jr. et al. in view of Reh et al. (U.S. Patent No. 5,538,262).

These rejections are respectfully traversed and reconsideration is respectfully requested.

Applicant has amended claim 36 to include "for growing a tissue part" to make it consistent with claims 30 and 57. Therefore, it is respectfully submitted that, since this phrase was previously presented in claims 30 and 57, this amendment does not raise any new issues and it is respectfully requested that the amendment be entered.

As noted previously, the term "substance" has been defined in the claims as "consisting of one of a tissue part, a scaffold having cells deposited thereon, and a scaffold including a tissue part thereon" (claims 30 and 36), and "consisting of at least one of a tissue part, a scaffold having cells deposited thereon, and a scaffold including one or more tissue parts thereon" (claim 57). Additionally, the term a "substance" is explained in paragraph two on page

1 of the entered substitute specification, specifically, "the tissue carrier and/or the tissue which is formed on it is designated as 'substance' in the following." Thus, the term a "substance" has three meanings: (i) a scaffold (being synonymous with a tissue carrier) having cells deposited thereon; (ii) a scaffold including a tissue part thereon; or (iii) a tissue part (in case a scaffold has disintegrated (or an existing tissue part has been used as scaffold)).

With regard to the rejections, applicant believes that a short discussion with regard to terminology may be beneficial, as was presented during prosecution in the parent application. Applicant respectfully submits that cellular engineering has been successful in understanding the biology of the cell and in growing and multiplying cells. With regard to human and animal tissue production, however, it was soon realized that merely multiplying the number of cells was not sufficient for growing a desired human or animal tissue in laboratory. The term **tissue** is generally defined as "a group or layer of cells that are alike and that **work together to perform a specific function** (see e.g. Appendix A2.), i.e. for growing tissues it is not sufficient to merely grow a large number of cells such as a clump of cells. In order to form a tissue, these cells must also work together to perform the specific functions of the desired tissue. It was only recently when **tissue engineering** started to develop that it has become feasible to grow tissues which can be implanted in the human or animal body as a replacement for diseased tissues like bone, cartilage, blood vessels, skin, liver etc. (see e.g. Appendix A1.) This success is strongly related to the use of newly developed tissue carriers, also called "tissue engineering scaffolds" or simply scaffolds.

The main functions of a **scaffold** are to provide mechanical support and stability, define and maintain a 3-D structure for the formation of new tissue of the desired form and size, and guide the development of the new tissue to achieve the appropriate functions. Ideally, the scaffold is biodegradable so that after implantation only the implanted tissue remains in the body. Scaffolds are a subject of intensive research since the functions of a scaffold are sophisticated and much more complex than the carriers used to assist in growing earlier cell cultures.

Cells of the specific tissue to be grown are embedded in a scaffold and the scaffold is then placed in a **bioreactor** allowing the cells to differentiate and construct the tissue in the

desired form and size. Bioreactor design must be suitable to provide the environment necessary for the cultivation of clinically sized implants. (See e.g. Appendix A3.)

With regard to the cited references, it is respectfully submitted that Starkie et al. disclose a device and method for the growth or multiplication of cells in a nutrient medium wherein the cells are kept floating in the medium (see e.g. Abstract). Dean Jr. et al. discloses a reactor and a reaction process for the growth or multiplication of cells in a fluidized bed consisting of biocatalyst beads. Both references are directed to a method or a process for the production of cells. The present invention, on the other hand, is not directed to the mere production of cells, but rather to the production of whole tissue parts having a specific form and function, such as, e.g., the production of coronary valves. In the method of the present invention, scaffolds (also called tissue carriers) or scaffolds with grown on tissue parts or tissue parts are held in flotation, i.e. the parts held in flotation are relatively large and heavy compared to the individual cells held in flotation in the teaching of e.g. Starkie et al. The problem solved by the present invention is completely different from the problem solved by the teachings in the cited art. The method of the present invention allows production of relatively large three-dimensional tissue parts. Previously known methods for the artificial production of tissue parts have been limited to flat, essentially two-dimensional structures.

Furthermore, it is respectfully submitted that a tissue or tissue part is more than a mere clump of cells. In a tissue or tissue part, i.e. a part consisting of tissue, the cells are differentiated and work together to perform a specific function (see e.g. the definition and explanation given above). The passage in Starkie's document (page 4, lines 21-25) cited in the Office Action discloses that clumps of cells may form in case of lymphoblastoid cells. This, however, demonstrates cell multiplication only. Starkie et al. do not give any hint on growing tissue or tissue parts. Starkie et al. do not disclose use of a scaffold for growing tissue parts.

Thus, with regard to the rejections of the claims in view of Starkie et al., it is respectfully submitted that claims 30, 36 and 57 are directed to methods and an associated bioreactor for tissue engineering. Claims 30, 36 and 57 include "for growing a tissue part". As discussed above, Starkie et al. are not directed to such methods and associated bioreactor and therefore, claims 30, 36 and 57 are allowable.

With regard to the rejection of the claims in view of Dean, Jr. et al., Applicant respectfully disagrees. It is respectfully submitted that Dean, Jr. et al. disclose "a reaction process for culturing cells, for example, for tissue culture and fermentation processes (see in the abstract.)" However, the description of the "biocatalyst beads" given in col. 4, lines 22-55 is directed to cultivating cells only, and apart from this, does not contain any information specific to tissue culture (further discussed below). Also in the examples disclosed by Dean, Jr. et al., only cell cultures for producing antibodies, and other protein products are described. It is therefore concluded that Dean, Jr. et al. merely discloses culturing cells except for the information that cultured cells may be used for tissue culture. This is, however, trivial, since anybody knows that tissue consists of cells.

In the Office Action the Examiner holds that the beads disclosed by Dean, Jr. et al. meet the instant claim language of "a scaffold". This is not correct. Claims 30, 36 and 57 include "for growing a tissue part". This clearly expresses that the claimed methods and associated bioreactor are directed to tissue engineering. As already discussed, the term "scaffold" has a very specific meaning in tissue engineering. The term "scaffold" e.g. includes providing a 3-D structure for the desired form and size of the tissue to be grown, guiding the development of the new tissue to achieve the appropriate functions (this is crucial since when cell differentiation goes wrong, only a clump of cells is formed!) and adjusting biodegradability so that after implantation only the implanted tissue remains in the body. None of these concerns are mentioned by Dean, Jr. et al. While the beads of Dean, Jr. et al. have a certain 3-D structure (see e.g. col. 4, lines 33-40), this 3-D structure is only adapted to cell growth, i.e. cell multiplication and not to the desired form and size of a tissue to be grown. The beads described in Dean, Jr. et al. have a size of 100 μm to 0.5 mm. This is much smaller than the scaffolds used in tissue engineering where the size of the scaffolds have the size of clinical implants.

Dean, Jr. et al. disclose a fluidized bed reactor containing a large volume of beads (see e.g. Figs 1-5 and col. 16, lines 20-36). Under these conditions the individual beads have virtually no space to move and touch each other continually. In the methods claimed in claims 30 and 57 of the present application, on the other hand, the scaffold is held in free flotation, thus

providing superior tissue growth conditions allowing uniform and undisturbed growth in all directions.

Accordingly, it is respectfully, submitted that claims 30, 36 and 57 are allowable in view of Dean, Jr. et al.

It is respectfully submitted that Freedman et al., Schoeb et al and Reh et al. do not make up for the lack of teaching in Starkie et al. and Dean Jr., et al.

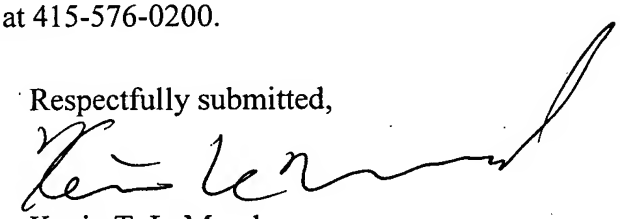
Claims 31-35, 37-56, 58-64 and 68-69 depend upon one of the allowable independent claims and therefore they are allowable for at least the reasons discussed above.

CONCLUSION

In view of the foregoing, Applicants believe all claims now pending in this Application are in condition for allowance and an action to that end is respectfully requested.

If the Examiner believes a telephone conference would expedite prosecution of this application, please telephone the undersigned at 415-576-0200.

Respectfully submitted,



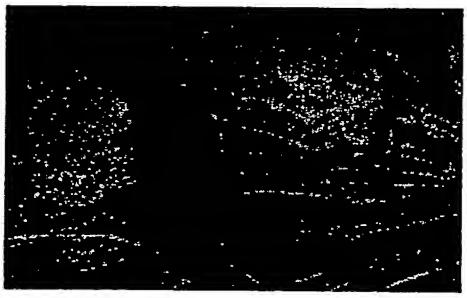
Kevin T. LeMond
Reg. No. 35,933

TOWNSEND and TOWNSEND and CREW LLP
Two Embarcadero Center, Eighth Floor
San Francisco, California 94111-3834
Tel: 415-576-0200
Fax: 415-576-0300
Attachments
KTL:lo
60280065 v1

TISSUE ENGINEERING

A1

Introduction



Tissue engineering involves the use of living cells to develop biological substitutes for tissue replacements. These can be utilized as opposed to the traditional synthetic implants. Loss of human tissue or organ is a devastating problem for a patient and family. Half of the annual health care cost in the United States is related to tissue or organ loss. Therefore, the goal is to design and grow new tissue outside the body that could then be transplanted into the body. Transplanting body parts has been medically used for quite some time, i.e., kidney transplants, but tissue engineering involves growing a tissue and than aiding in transplant. Using this technology, it will one day be possible to regenerate or replace damaged tissues with laboratory-grown parts such as bone, cartilage, blood vessels, and skin.

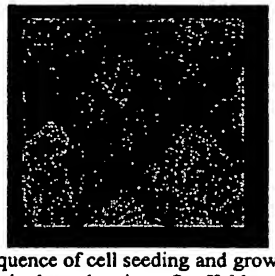
The tissue engineering field is barely a decade old. Thus far in its development there has been one form of man-made skin which is already on the market in the U.S. Tiny tubes containing cells that secrete painkilling substances have been implanted into the spinal columns of people with chronic pain. And tissue-engineered cartilage is in clinical tests and is expected to be commercially available within the next few years. Scientists have learned how to cultivate human embryonic stem cells that might allow researchers to build custom-made organs on demand.

The term 'tissue engineering' was officially coined at a National Science Foundation workshop in 1988 to mean the application of principles and methods of engineering and life sciences toward fundamental understanding of structure-function relationships in normal and pathological mammalian tissues and the development of biological substitutes to restore, maintain or improve tissue function. Although cells have been cultured outside the body for many years, research has recently begun to develop complex three-dimensional tissue constructs that will ideally mature into fully functional tissues and organs.[1]

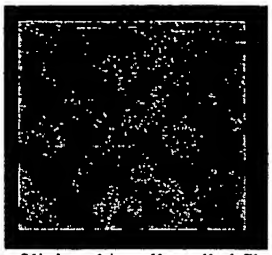
There are now several products that are commercially available and some of the products are seen here:[2]

Product	Distributor / Manufacturer	Details
Apligraf	Novartis / Organogenesis	Approved by the FDA on May 26, 1998. This product has both a dermal and epidermal layer. Initial USA indication is venous stasis ulcers. For more information, visit http://www.organogenesis.com
Dermagraft	Smith & Nephew / ATS	Dermal tissue layer. Initial USA indication applied for is full thickness diabetic foot ulcers.
Dermagraft TC	Smith & Nephew / ATS	This product is approved for sale in the USA for burn wounds. You would not substitute Dermagraft TC for Dermagraft. They are two very different products with distinct indications.

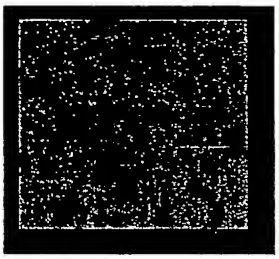
Skin is the most commonly produ)in Tissue Engineering:



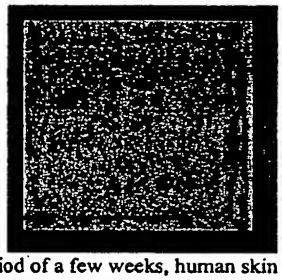
The sequence of cell seeding and growth can be seen in these drawings. Scaffolds are designed for specific tissues; this one is for skin.



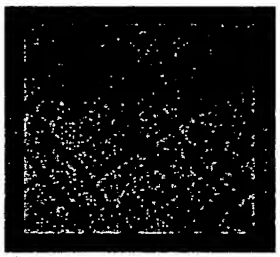
Millions of living skin cells, called fibroblasts, are seeded within the scaffold. The cells multiply on the scaffold, which is contained and nourished within a device called a bioreactor.



In the bioreactor, the cells grow and multiply. As they organize themselves into three-dimensional layers of skin, the scaffold material slowly degrades and disappears.



Over a period of a few weeks, human skin tissue is formed, ready to treat victims of serious burns.



This photograph shows skin cells that have multiplied. The cells have been tricked into thinking they are in their natural environment instead of an engineered structure. Several days after seeding, they completely fill in the space of the scaffold. A few weeks of further growth will produce a piece of artificial skin large enough to be used for healing burns or replacing diseased tissue.

Courtesy of Advanced Tissue Sciences, Inc.[3]

There are several tissue engineering companies that have emerged recently including Sulzer Medica, Life Cell, Advanced Tissue Sciences, Organogenesis, Genzyme Tissue Repair, and Integra Life Sciences.[4]

References:

1. http://biomed.tamu.edu/biomaterials/TissueEngineering.htm
2. http://www.medicaledu.com/tissue%20engineering.htm
3. Taken from http://www.eweek.org/2000/nbm/connect/index.htm
4. www.che.utexas.edu/~schmidt/links/eng.html

Created by Christine Brown and Jessica Laclair for Biochemical Engineering Fall 2000

A2

thyroid-stimulating hormone

TSH. A hormone produced by the pituitary gland. TSH stimulates the release of thyroid hormone from thyroglobulin. It also stimulates the growth of thyroid follicular cells. An abnormal TSH level may mean that the thyroid hormonal regulation system is out of control, usually as a result of a benign condition (hyperthyroidism or hypothyroidism).

thyroidectomy (thigh-roid-EK-toe-mee)

Surgery to remove part or all of the thyroid.

tiazofurin

An anticancer drug being studied to stop cell growth.

time to progression

A measure of time after a disease is diagnosed (or treated) until the disease starts to get worse.

tin ethyl etiopurpurin

An anticancer drug that is also used in cancer prevention. It belongs to the family of drugs called photosensitizing agents. Also called SnET2.

tin Sn 117m DTPA

A radioactive chemical being studied to treat bone pain associated with cancer.

tinidazole

A drug used to treat protozoal infections, such as amebiasis, giardiasis, and trichomoniasis. It belongs to a family of drugs called antiprotozoal agents. Tinidazole is also being evaluated in the treatment of Helicobacter pylori infections in people with low-grade gastric lymphoma.

tipifarnib

An anticancer drug that inhibits the transformation of normal cells to cancer cells. It belongs to the family of drugs called enzyme inhibitors. Also called R115777.

tirapazamine

A drug that makes tumor cells more sensitive to radiation therapy.

tissue (TISH-oo)

A group or layer of cells that are alike and that work together to perform a specific function.

tissue flap reconstruction

A type of breast reconstruction in which a flap of tissue is surgically moved from another area of the body to the chest, and formed into a new breast mound.

TLK286

A substance that is being studied as a treatment for cancer. It belongs to the family of drugs called glutathione analogs.

TM

Transcendental meditation. A mental technique used to promote relaxation, reduce stress, and improve quality of life.

TNF

Tumor necrosis factor. A type of biological response modifier (a substance that can improve the body's natural response to disease).

TNM staging system

A system for describing the extent of cancer in a patient's body. T describes the size of the tumor and whether it has invaded nearby tissue, N describes any lymph nodes that are involved, and M describes metastasis (spread of cancer from one body part to another).

TNP-470

A drug that belongs to the family of drugs called angiogenesis inhibitors. It prevents the growth of new blood vessels into a solid tumor.

tocladesine

A substance that is being studied as an anticancer drug. It is an analogue of a substance that occurs naturally in the body (cyclic adenosine monophosphate).

tomography (toh-MAH-gra-fee)

A series of detailed pictures of areas inside the body; the pictures are created by a computer linked to an x-ray machine.

tonsils

Small masses of lymphoid tissue on either side of the throat.

topical

A3

CHAPTER 11

TISSUE CULTURE BIOREACTORS: CHONDROGENESIS AS A MODEL SYSTEM

Lisa E. Freed and Gordana Vunjak-Novakovic

INTRODUCTION

A tissue engineering system based on isolated cartilage cells (chondrocytes), biodegradable polymer scaffolds, and tissue culture bioreactors has been used for in vitro cartilage growth (chondrogenesis) (Fig. 11.1).[1] The scaffold defined construct shape and dimensions, permitted cellular differentiation, and biodegraded in parallel with the accumulation of tissue components.[2] Bioreactors provided mixing which significantly improved the yield and spatial uniformity of cell seeding and increased the rates of cell proliferation and tissue regeneration.[3-6] Constructs grown in vitro resembled normal cartilage histologically and biochemically[3-6] and continued to remodel following in vivo implantation, subcutaneously in nude mice[7,8] and intra-articularly in rabbits.[9]

The specific requirements of cartilage tissue cultivation include: (1) a three-dimensional (3-D) scaffold that allows spatially uniform cell attachment in conjunction with the maintenance of cell phenotype, (2) adequate mass transfer rates of gases and nutrients, and (3) an in vitro culture environment that is permissive for chondrogenesis, or even designed to promote selected cell functions. The in vitro morphogenesis of engineered tissues is expected to depend on flow and mixing during cultivation in at least two ways: by direct hydrodynamic effects on cell shape and function and by flow-induced changes in mass transfer rates. Bioreactor design considerations for the cultivation of clinically sized implants (i.e., cartilage 2-5 mm thick) must thus include the interactions between the growing tissue construct and its fluid dynamic environment.

Identification and control of key parameters that determine in vitro chondrogenesis are essential for cartilage tissue engineering, since the required size, shape, biochemical composition and morphology of cartilage implants may vary from one case to another. Ideally, for a given clinical application (e.g., the repair of fibro- or articular cartilage), culture conditions can be optimized with respect to the cell source, polymer

Principles of Tissue Engineering, edited by Robert Lanza, Robert Langer and William Chick.

Fig. 11.1. Model system for cartilage tissue engineering: Isolated chondrocytes from harvested cartilage are seeded on a biodegradable polymer scaffold and cultured in vitro prior to in vivo implantation, e.g., to repair articular cartilage.

scaffold, medium composition and exchange rate and bioreactor fluid dynamics.

The long-term goals of our studies are an improved understanding of tissue morphogenesis and the in vitro cultivation of tissue constructs with a range of defined sizes and shapes for a variety of clinical applications. In this chapter, we discuss the scientific and practical issues related to the design and operation of bioreactors for the cultivation of large, functional tissue constructs using cartilage as a model system.

CARTILAGE TISSUE ENGINEERING

CARTILAGE

Articular cartilage was harvested aseptically from the femoropatellar grooves of knee joints from 2-3 week old bovine calves. The tissue was either chopped into 2 mm^3 cubes for cell isolation or formed into 5 mm diameter x 2.5 mm thick discs with flat surfaces using a cork borer, a vacuum holder and a scalpel.

CELL ISOLATION

Chondrocytes were isolated by digestion with type II collagenase as previously described.[7] Cells were resuspended in culture medium (Dulbecco's Modified Eagle Medium), containing 10% fetal bovine serum, 10 mM N-2-Hydroxyethyl-piperazineN'-2-ethane-sulfonic acid, 0.1 mM nonessential amino acids, 0.4 mM proline, 50 mg/L ascorbic acid, 100 U/cm^3 penicillin, and 100 µg/cm^3 streptomycin.

POLYMER SCAFFOLDS

Polyglycolic acid (PGA) scaffolds were made as previously described.[2] PGA was extruded into 12 µm diameter fibers, processed to form a 96-97% porous nonwoven mesh with a bulk density of 44-62 mg/cm^3, die punched into discs (5-10 mm diameter x 2-5 mm thick) and sterilized with ethylene oxide.

TISSUE CULTURE

Two bioreactor systems currently under investigation are shown in Figure 11.2.[4,5] Prior to cell seeding, PGA scaffolds were prewetted in culture medium, threaded onto needles, positioned using silicone tubing, and fixed to a stopper placed in the mouth of a spinner flask. The flasks were filled with 120 cm^3 of culture medium and placed in a humidified 37°C/5% CO_2 incubator 8-12h prior to cell inoculation with the side arm caps loosened to permit gas exchange. The flasks were mixed using a magnetic stir bar at 50-80 rpm and inoculated with freshly isolated chondrocytes (4-5 x 10^5 cells per cm^3 culture medium).

After 3 days, the constructs were cultured in either static flasks, in flasks stirred magnetically at 50-80 rpm, or in rotating vessels. In rotating vessels, gas exchange was achieved by pumping filter sterilized incubator air through the central membrane.[10] Constructs were freely suspended by rotating the entire vessel around its central axis and the rotation speed was gradually increased from 15 to 28 rpm in order to compensate for increasing construct mass.[4] In all vessels, culture medium was replaced batchwise, at a rate of 50-100% every 2-3 days over 4-8 wks of cultivation and constructs were sampled at timed intervals for structural and functional analyses.

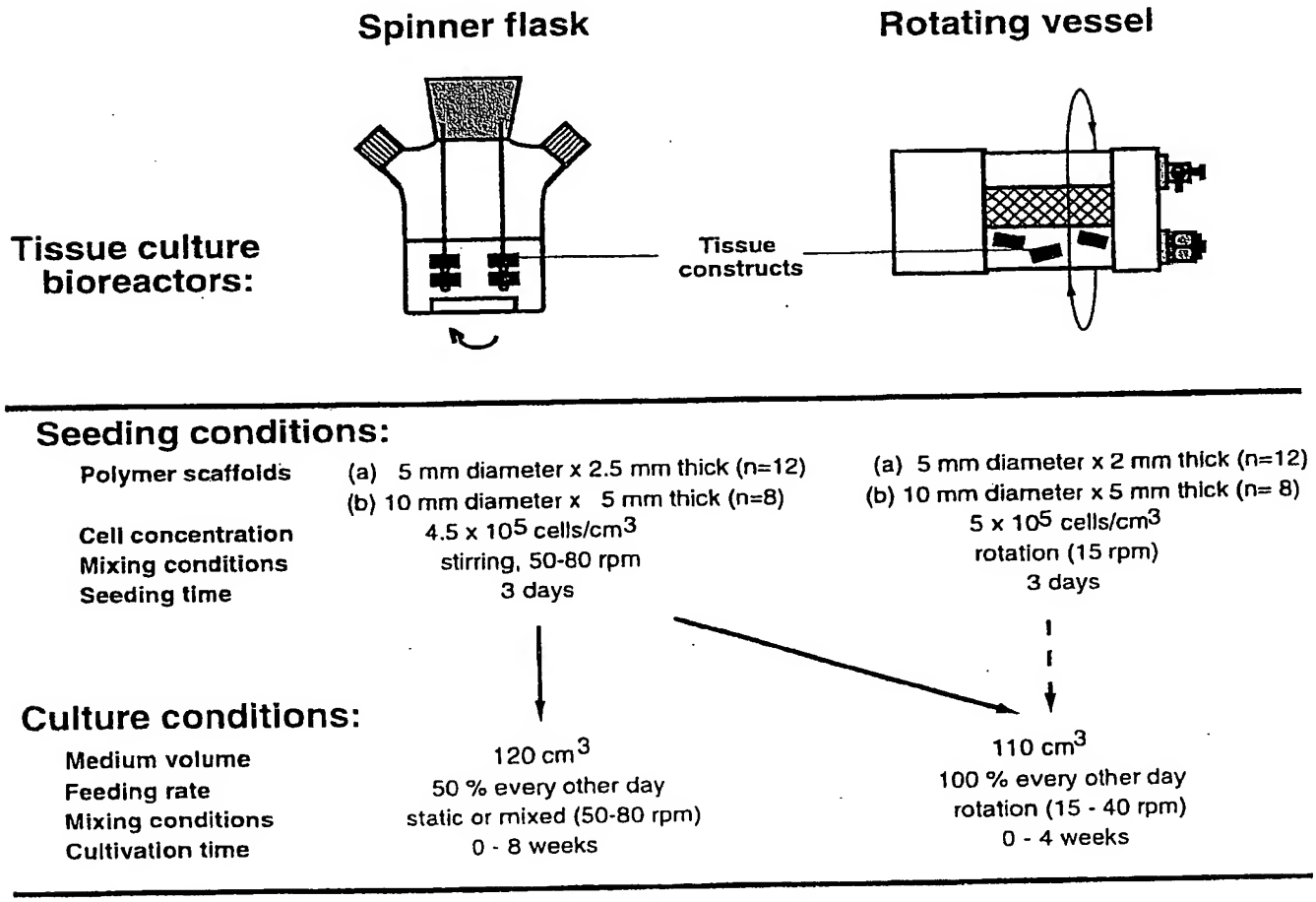
Construct Analyses

Samples for histological analyses were embedded in paraffin, cross-sectioned (5 μm thick), and stained with hematoxylin and eosin (H&E) for cells and safranin-0 for glycosaminoglycan (GAG).[11] Construct dimensions and capsule thicknesses were measured with an inverted microscope, a video camera, a frame grabber, and commercially available image analysis software.[4] Samples for biochemical analyses were frozen, lyophilized, and papain digested.[7] The number of cells per construct was assessed from DNA content, measured spectrofluorometrically, using the conversion factors 7.7 pg DNA per chondrocyte and 10^{-10} g dw per chondrocyte.[2,12] The amount of sulfated GAG was determined spectrophotometrically using bovine chondroitin sulfate as a standard.[13] The total collagen content was determined from the hydroxyproline content, measured spectrophotometrically, using a hydroxyproline to collagen conversion factor of 0.143.[14]

Fluid-Dynamic Studies

A perfused rotating bioreactor was custom designed for mixing studies (Fig. 11.3a). The 120 cm^3 vessel, which consisted of the annular space between two concentric cylinders with diameters of 5.75 and 2 cm, was simultaneously perfused and rotated around its central axis. Fluid entered and exited from the vessel via rotating adapters that were con-

Fig. 11.2. Tissue culture bioreactors: cell seeding and cultivation conditions used for spinner flasks and rotating vessels.

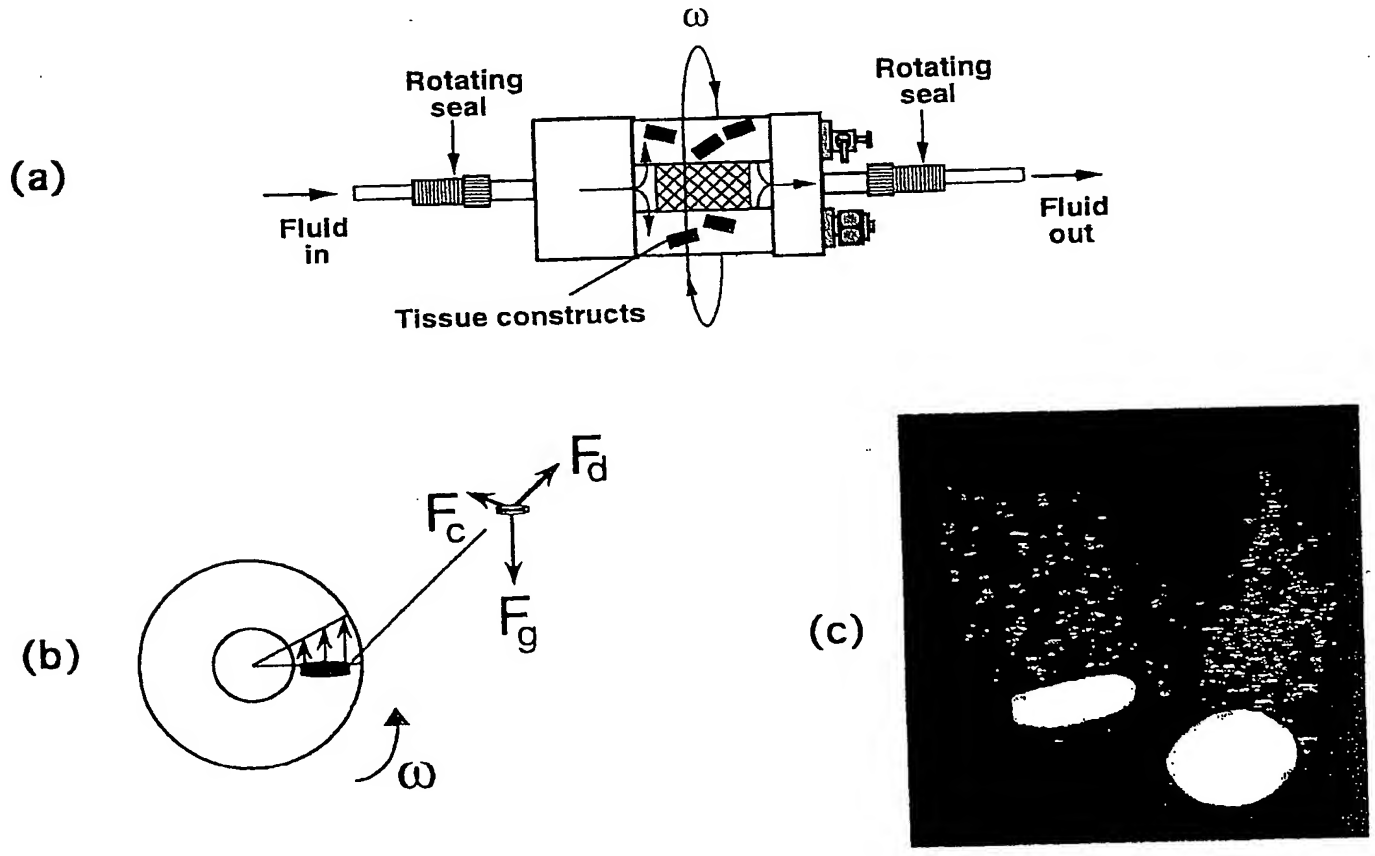


nected to six 0.1 cm diameter holes, which were positioned circumferentially at each end of the rotating central cylinder.

Flow-visualization and residence time distribution (RTD) studies were done using vessels containing water and n = 12 cartilage discs (5 mm diameter x 2.5 mm thick). The discs were maintained freely suspended within the vessel by adjusting the rotation rate (Fig. 11.3b). Flow patterns were visualized using atomized aluminum powder with an average diameter of 29 μm (2 cm^3 of a dense slurry), fluorescein dye (2 cm^3), and a fiber-optic light. The fluid streamlines and vortices associated with the settling discs in the rotating flow field were qualitatively assessed from videotapes (Fig. 11.3c).

RTD studies were done using a solution of blue dextran in water (1 g/L, 2 x 10^6 D molecular weight) as a tracer dye. The vessel was simultaneously perfused with tracer at 3 cm^3/min and rotated around its central axis at 20 rpm. Separate RTD studies were done in a 6.5 cm diameter spinner flask containing 120 cm^3 water and n=12 cartilage discs that were fixed in place as described above for cell culture studies. The flask was simultaneously perfused with tracer dye at a rate of 3 cm^3/min and stirred at 90 rpm. Tracer concentrations were measured spectrophotometrically at 620 nm in samples obtained from the outlets of both bioreactors at timed intervals (40-50 samples over 6-12 residence times). Experimental conditions corresponded to a closed-closed vessel.[15]

Fig. 11.3. Fluid-dynamic studies: (a) side view of the prototype rotating, perfused bioreactor, (b) cross-section of the vessel showing a suspended disc in the rotational flow field, (c) photograph of vortex shedding by the suspended disc.



The data were first tested for consistency by closing the material balance for tracer at the reactor outlet. From the tracer concentration profile, F(t), the response curve, E(t), was obtained and used to calculate the nondimensional dispersion, $^{o}\theta^{2}$ (Levenspiel[15] p. 261, Eqs. 11 and 12). Mixing in the liquid phase was assessed by calculating the Peclet number, D/uL, using the axial dispersion model and the number of stirred tanks in series, N, using the compartment model (Levenspiel[15] p. 276, Eq. 36 and p. 291, Eq. 51c, respectively).

BIOREACTOR FLOW CONDITIONS

Flow and mixing conditions in spinner flasks and rotating vessels are compared in Table 11.1. In flasks stirred at 50 rpm, tissue constructs were exposed to turbulently mixed fluid at an impeller Reynolds number of 1370; fluid flow at the construct surface was characterized by eddies, the smallest of which (Kolmogorov eddies) had an estimated size of 250 μm and velocity of 0.39 cm/s.[5] The average intensity of turbulence in the spinner flasks was below the level previously shown to cause detachment of mammalian cells,[16,17] but was sufficient to stimulate chondrocytes at the construct surface to flatten, proliferate, and form a fibrous capsule.

In rotating vessels, neither the entire fluid mass, which rotated as a solid body around the vessel spin axis, nor the low axial flowrate of 3 cm³/min caused any significant mixing. Fluid mixing was generated

Table 11.1. Flow and mixing conditions in bioreactor vessels

	Spinner flask	Rotating vessel
(a) Construct cultivation (batch system)		
Vessel volume (cm^3)	120	110
Stirring or rotation rate (rpm)	50–80	15–28
Number of constructs per vessel	8	12
Construct diameter (cm)	0.95–1.1	0.5 - 0.74
Construct thickness (cm)	0.3–0.5	0.2 - 0.37
Fluid flow pattern	Turbulent flow of stirred fluid around constructs that are fixed in place	Oscillatory settling of constructs that are freely suspended in the rotational flow field
Relative fluid - construct velocity	Function of the axial and radial position of a construct	Function of construct properties, approximates its settling velocity (2 - 3 cm/s)
Characteristic parameters of fluid flow at the construct surface	Impeller Re number[1]: 1370 Size of smallest eddies[1]: 250 μm Velocity of smallest eddies[1]: 0.39 cm/s	Settling Re number[2]: 114 - 207 Moment of inertia[2]: $I^* = 0.023$ Average shear stress[2]: 1.5 dyn/cm^2
(b) Mixing studies (continuous flow system)		
Vessel volume (cm^3)	120	120
Fluid flowrate (cm^3/min)	3	3
Stirring or rotation rate (rpm)	90	20
Number of constructs per vessel	12	12
Construct dimensions (diameter x thickness, cm)	1.0 x 0.25	0.5 x 0.25
Nondimensional dispersion, σ_θ^2	0.900	0.918
Peclet number, D/uL	3.1	3.8
Number of mixed tanks in series, N	1.1	1.1

(1) data from Vunjak-Novakovic et al.[5]
(2) data from Freed and Vunjak-Novakovic[4]

by the settling tissue constructs (Fig. 11.3 b-c). Fluid dynamic conditions were characterized by a construct settling velocity of 2-3 cm/s, settling Reynolds numbers of 114-207 and a dimensionless moment of inertia of 0.023.[4] Settling was associated with oscillations, tumbling, wake formation and vortex shedding.[18] Each settling disc thus mixed the liquid in its immediate environment, and convective mixing in the vessel as a whole resulted from wake-wake collisions and axial disc translation.[19] RTD studies demonstrated that spinner flasks and rotating vessels were well mixed under the selected operating conditions. Calculated values of σ_θ^2 (0.900 and 0.918), D/uL (3.1–3.8), and N (1.1) all indicate that mixing in both bioreactors closely approximated that in a perfectly mixed vessel (Table 11.1).

EFFECTS OF MIXING ON CONSTRUCT STRUCTURE

Mixing affected construct size, shape and structure as follows. Constructs cultured in static flasks were thin, had rough surfaces and appeared irregular while those from mixed flasks were thick, had smooth surfaces and maintained the dimensions of the original polymer scaffold (Figs. 11.4 a-b, respectively). Histologically, statically grown constructs consisted of round cells embedded in a nonhomogeneous cartilaginous matrix while mixing induced the formation of a surface capsule around an inner tissue phase (Figs. 11.4 c-d, respectively).

The capsule consisted of multiple layers of flat cells, contained collagen but little GAG, and was approximately 300 μm thick after 8 weeks of cultivation.[5] Natural articular cartilage has an analogous, 25 μm thick surface zone (or capsule) containing flat cells and collagen but little GAG.[20] Capsule presence increases the stiffness and decreases the permeability of natural cartilage.[21] Hydrodynamic forces can thus potentially be utilized to control capsule thickness and thereby construct biomechanical and diffusional properties.

Mixed culture conditions were associated with increased rates of cell proliferation and extracellular matrix (ECM) regeneration. In particular, constructs grown in mixed cultures contained up to 50% more cells (Fig. 11.5a), 60% more GAG (Fig. 11.5b), and 125% more collagen (Fig. 11.5c) than constructs grown statically.[5] The effective rate of nutrient supply to cells within constructs depends on mixing intensity and diffusional permeability of a construct which is in turn determined by its structure.[22] Improved tissue regeneration in mixed cultures can thus be attributed in part to increased mass transfer rates at the construct surface.

COMPARISON OF ENGINEERED WITH NATURAL CARTILAGE

Rotating vessels can be used to cultivate tissue engineered cartilage 7-8 mm in diameter x 3-4 mm thick within 2-3 weeks.[6] These constructs resembled explants of natural cartilage with respect to morphological appearance and biochemical composition. Both engineered and natural cartilage had high, spatially uniform distributions of lacunae containing round cells within a compact ECM that stained strongly positive for GAG, and thin surface layers of flat cells (Fig. 11.6). Biochemically, constructs consisted of cells (7% dw), GAG (30% dw), collagen (19% dw), and water (88% of total wet weight) (Fig. 11.7). As compared to average values measured for the harvested bovine calf cartilage, constructs contained 50% more cells, 19% less GAG, 55% less collagen and 18% more water.

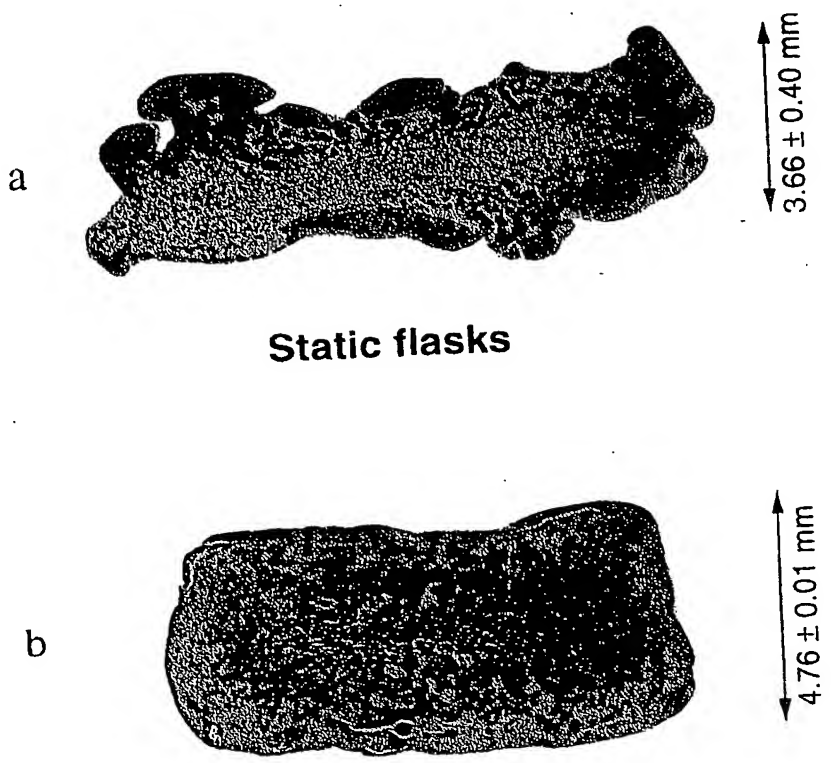


Fig. 11.4. Histological cross-sectional of constructs grown in spinner flasks: (a & c) static culture, (b & d) mixed culture (original magnification x1 for a&b and x100 for c&d, safranin-O stain). Fig 11.4 c & d appear on opposite page.

SUMMARY AND FUTURE DIRECTIONS

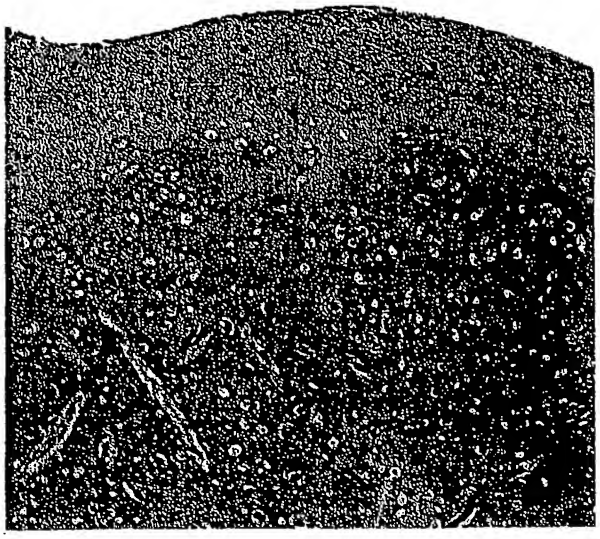
Bioreactors can be used in conjunction with isolated cells and 3-D biodegradable polymer scaffolds to cultivate cartilage implants for potential clinical use. The design and operation of two tissue culture bioreactors, spinner flasks and rotating vessels, are presented here in detail. Other bioreactor designs are also under investigation, such as continuously perfused 1.5 cm^3 volume cartridges.[23] In vitro grown cartilage constructs closely resembled natural cartilage histologically and biochemically. It is likely that with further chondrogenesis (in vitro and/or in vivo), the structure and function of engineered tissue could eventually reach those of natural cartilage. However, several basic and practical problems still need to be resolved before bioreactors can be routinely used for the cultivation of cartilage and other clinically useful tissues.

Cell Source and Scaffold Seeding

A defined cell source and optimization of cell seeding methods are required due to the limited availability and variability of donor cells. In addition, high initial construct cellularities are desirable since they were associated with high rates of cartilage matrix regeneration,[3] presumably due to cooperative cell-cell and cell-matrix interactions.[24,25] The development of reliable methods to source, amplify and cryopreserve human chondrocytes remains a critical issue. Cells can be isolated either from autologous cartilage and amplified in vitro[26] or from al-

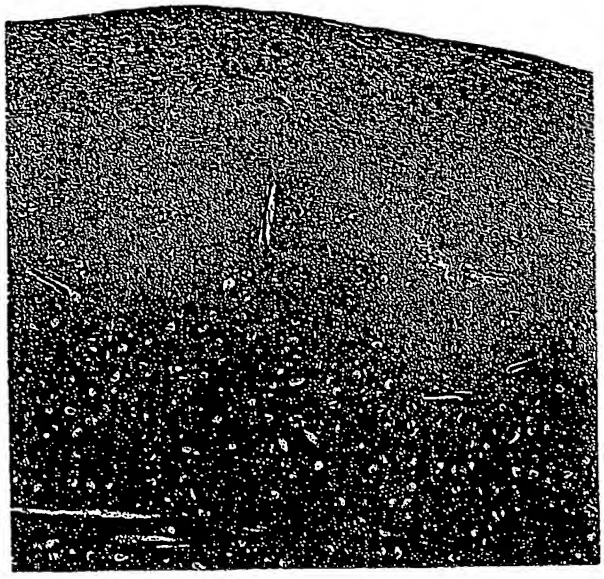
Fig. 11.4. (continued)

c



Static flasks

d

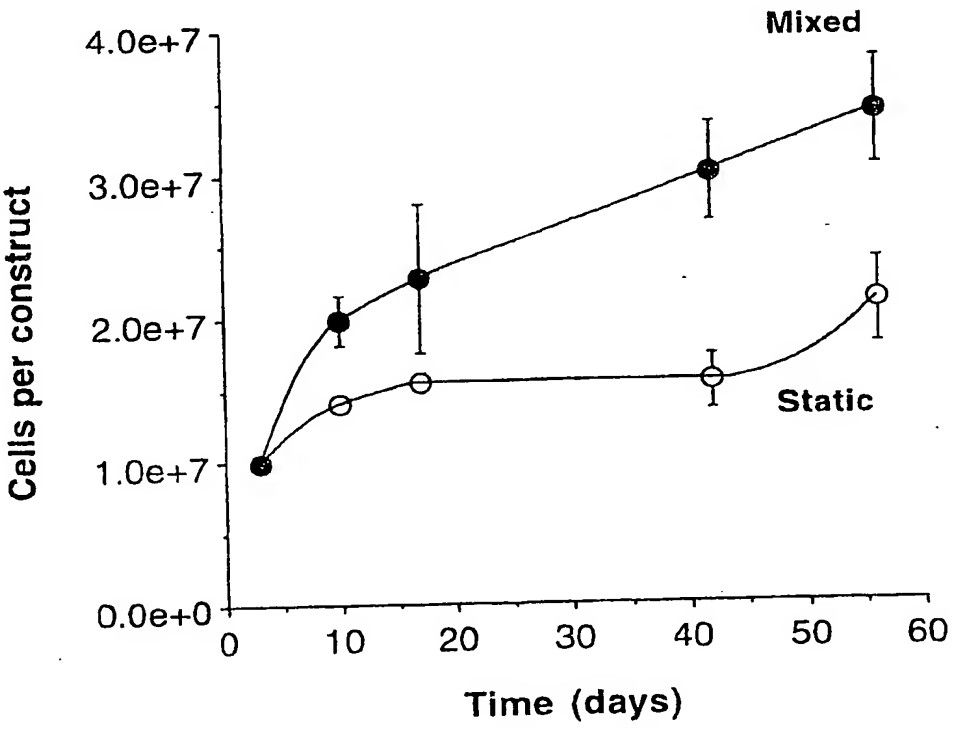


Mixed flasks

lograft cadaver cartilage. Alternative cell sources include embryonal cells, which are immunoprivileged and have a higher mitotic potential than chondrocytes from adult donors, and bone marrow stromal fibroblasts, which can be induced to express a cartilaginous phenotype.[27]

During cell seeding, convection causes cells to penetrate the scaffold and inertial impacts between the cells and the polymer fibers result in cell attachment.[1] For efficient and spatially uniform cell seeding, the bioreactor must provide both a uniform cell suspension and relative velocity between the cells and the fibers. These design requirements can be met using either spinner flasks or rotating vessels. Chondrocytes were seeded most efficiently in the spinner flask system

Fig. 11.5. Tissue regeneration kinetics in static and mixed flasks: (a) cells per construct, (b) GAG (% dry weight) and (c) collagen (% of dry weight). Data represent the average ± SD of 6 independent measurements. Figure 11.5c is on the top of the opposite page.



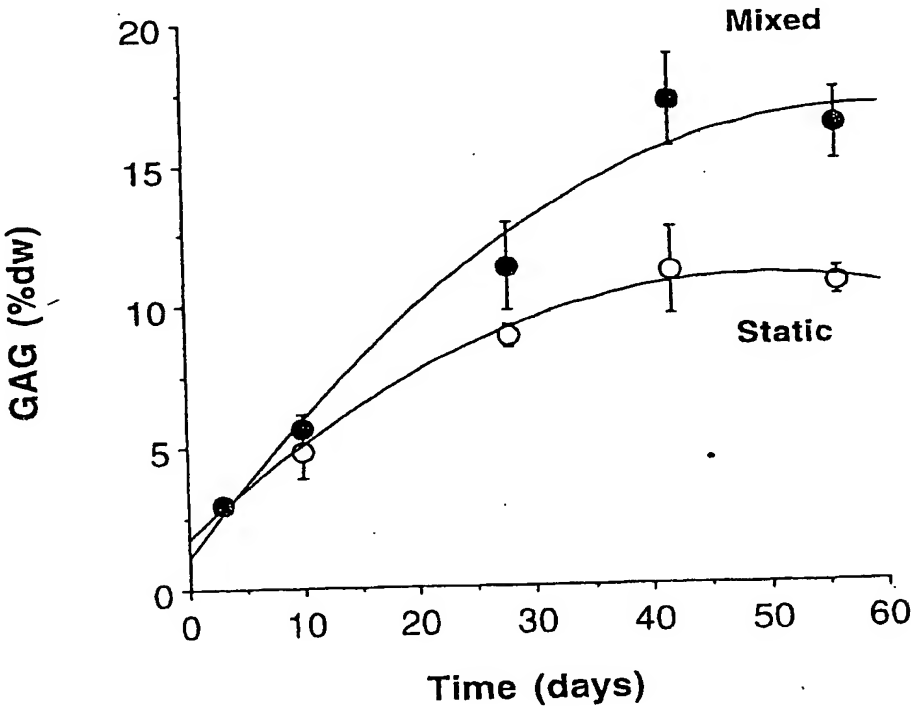


Fig. 11.5. (continued)

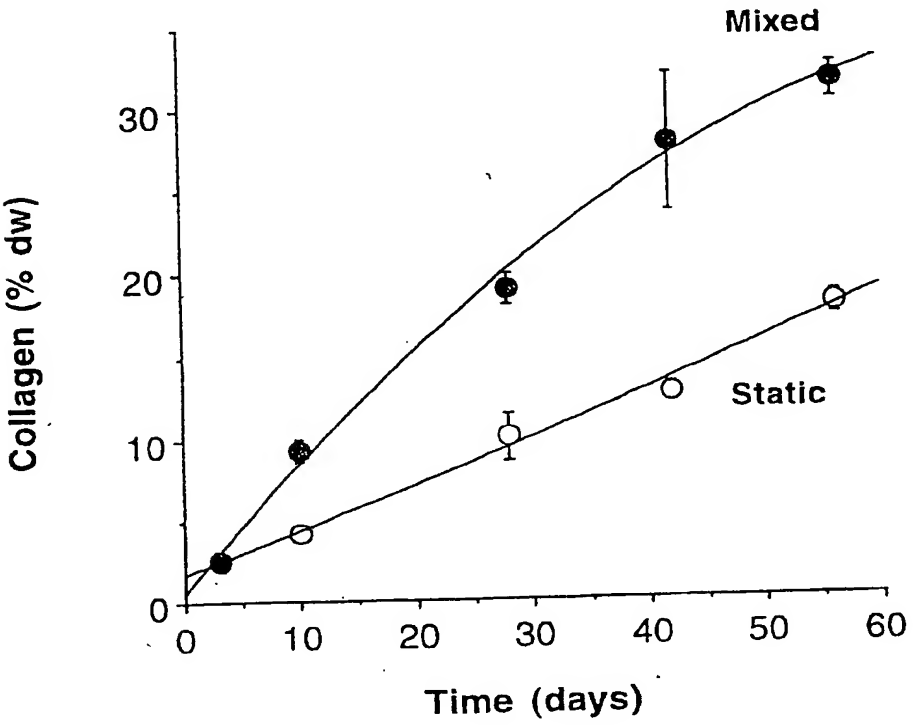
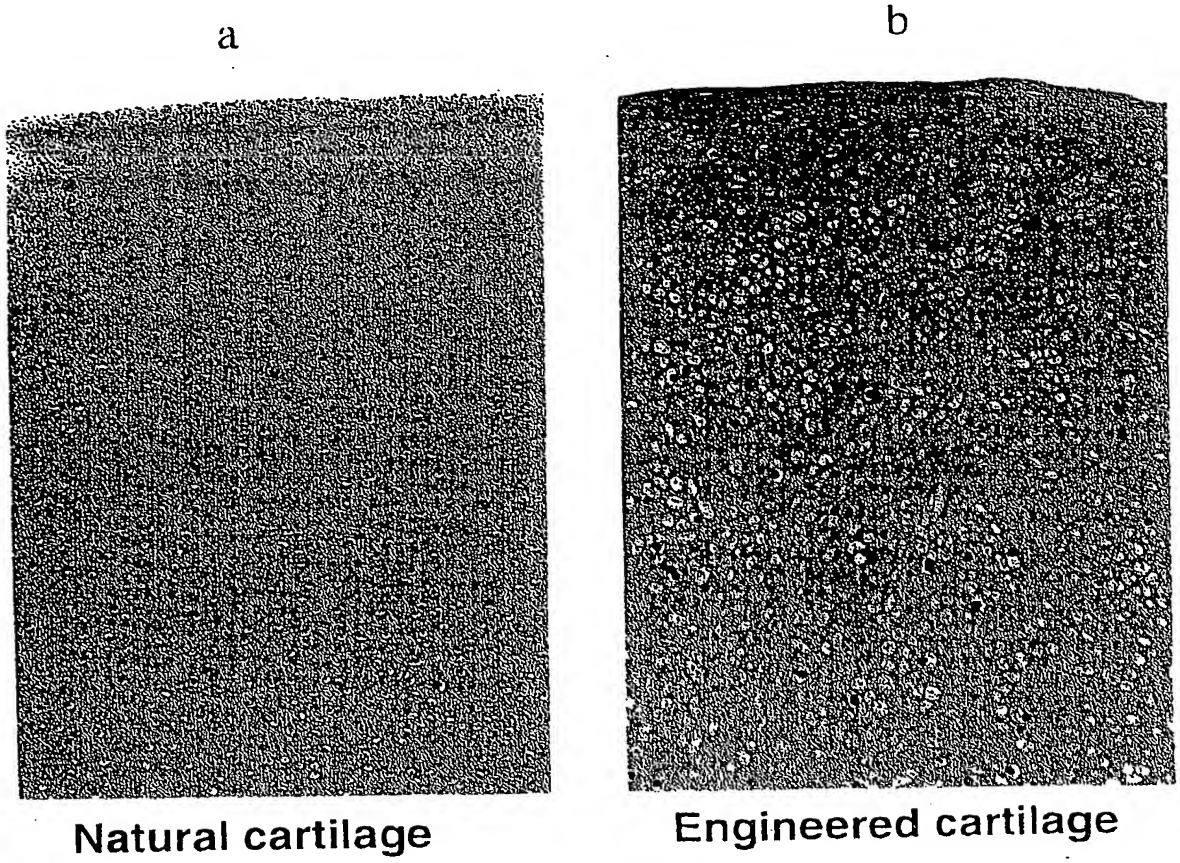


Fig. 11.6. Histomorphologies of natural and engineered cartilage: (a) natural cartilage and (b) engineered cartilage after 24 days in a rotating vessel (original magnification x100, safranin-0 stain).



in which the scaffolds were fixed in place apart from each other and exposed to unidirectional flow which resulted in convective motion of the suspended cells into the scaffold interior. Alternatively, more shear-sensitive cells can be seeded in rotating vessels as long as the number of freely suspended scaffolds per unit volume of culture medium is relatively small.

The In Vitro Culture Environment

In vitro culture conditions need to be optimized in order to custom-engineer tissue constructs for specific clinical applications (e.g., the repair of fibro- or articular cartilage). An integral part of this goal is to establish design criteria for the constructs themselves, i.e., required dimensions and structural and functional properties that may vary from one application to another. Following in vivo implantation, the engineered tissue should ideally achieve the biochemical composition and biomechanical properties of natural cartilage and fully integrate into the host tissue. Chondrogenesis in cell-polymer constructs is known to continue in vivo[7,9] and can be enhanced by continuous passive motion.[28] Further studies are needed to (a) measure construct biomechanical properties and (b) correlate construct histomorphology, composition and biomechanics at the time of implantation with the course of in vivo chondrogenesis.

For the cultivation of 3-D constructs, the entire surface of the growing tissue should be exposed to well-mixed medium in order to minimize diffusional constraints. Mixing both maintains a uniform concentration of chemical species (e.g., pH, gases and nutrients) in the bulk phase and increases the mass transfer rate at the construct surface. The results of RTD studies indicate that the above mixing requirements can be met using either spinner flasks or rotating vessels.

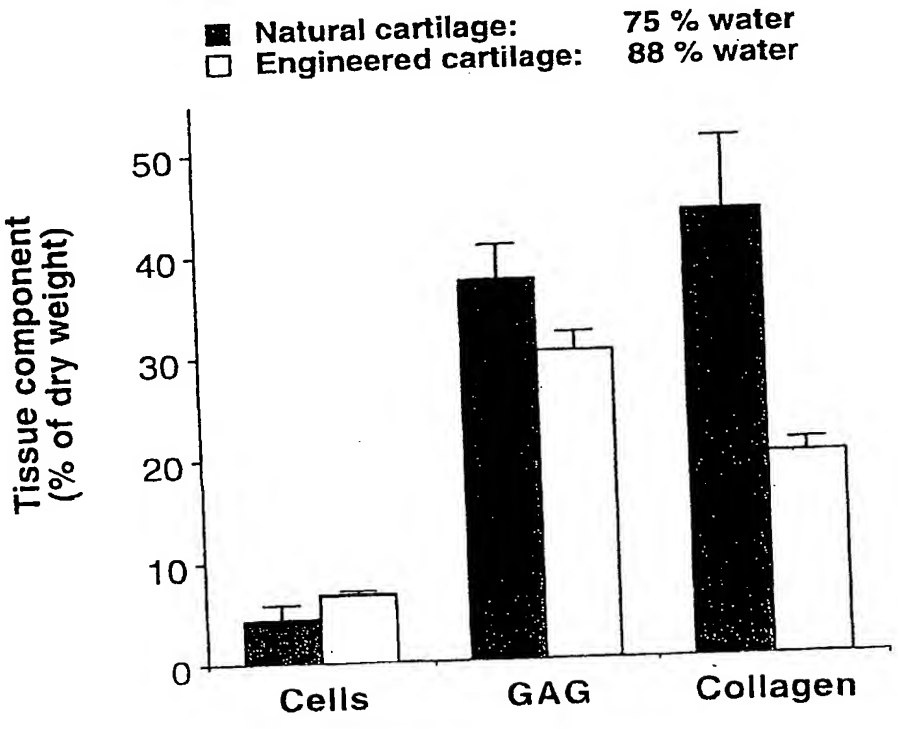


Fig. 11.7. Biochemical compositions of natural and engineered cartilage: amounts of water (% wet weight) and tissue components (cells, GAG and collagen, % of dry weight). Data represent the average ± SD of 6 independent measurements.

In addition, mixing-induced pressure and velocity fluctuations at the construct surface are likely to stimulate in vitro tissue growth. Previous studies have shown that in vivo, tissue morphogenesis is influenced by environmental forces[29] and in vitro chondrogenesis is stimulated by dynamic loading.[30,31] In the case of tissue engineered cartilage, mixed culture conditions improved construct structure and composition.[5,6]

Bioreactor Design and Operation

A closed bioreactor system that continuously supplies nutrients and automatically controls tissue culture parameters according to the changing needs of the growing constructs would be ideal for the cultivation of tissues for actual clinical use. Based on the results of fluid-dynamic and tissue culture studies, the envisioned bioreactor design embodies a rotating vessel with a membrane oxygenator for gas exchange and a continuous flow system for medium exchange (Fig. 11.3a). Specification of vessel geometry and operating conditions will be based on exact metabolic requirements during the formation of cartilage or other engineered tissues. Appropriate rates of gas and nutrient exchange for optimal tissue growth should thus be known as a function of construct size and composition over the course of cultivation. The prototype perfused vessel must meet the metabolic needs of the cells while maintaining the performance demonstrated for batch tissue culture systems, which is likely since the perfusion rate that was associated with excellent mixing in RTD studies (3cm^3/min or 4.3 L/day) was about 100-fold higher than the current refeeding rate of batch cultures (30-60 cm^3/day, Fig. 11.2). Finally, automated control systems, which might include biosensors to trigger appropriate changes in the rates of vessel rotation and medium perfusion, are needed to maintain optimal levels of gas and nutrient over the course of in vitro cultivation.

Scale-up to Larger Constructs and Extension to Other Cell Types

The resurfacing of an entire joint (e.g., a knee) would require a cartilage construct approximately 5 cm in diameter by 1-5 mm thick; reconstructive surgery applications might require even thicker constructs. This implies a need to develop larger bioreactors in a variety of geometrical configurations. One possible design is a rotating bioreactor with a high aspect ratio[32] which has been used to cultivate cell-polymer tissue constructs up to 10 cm in diameter by 2 mm thick (Freed et al, unpublished data). In addition, the surface area for gas exchange per unit of cell culture volume is more than 5-fold higher in the high aspect ratio vessel than in the rotating vessel shown in Figure 11.2, which should permit the in vitro growth of tissues with high oxygen requirements (e.g., cardiac or neural tissue).

In summary, tissue engineering represents a new therapy in which cell-based implants grown in vitro can potentially be used to repair damaged cartilage as well as a variety of other tissues (e.g., skin, bone, blood vessels, liver).[33] Bioreactors were shown to represent a simple and relatively straightforward system for the cultivation of cartilage constructs with morphological and biochemical properties that approach natural tissue. Future studies will focus on optimizing bioreactor design and extending the approaches and methodologies developed for cartilage tissue engineering to other cell-polymer systems.

Acknowledgments

This work was supported by the National Aeronautics and Space Administration (grant NAG 9-655) and Advanced Tissue Sciences (La

Jolla, CA). The authors thank R. Langer for his critical review of this manuscript, and V. Jackson and C. Villacorta for the technical help.

REFERENCES

1. Freed LE, Vunjak-Novakovic G. Tissue engineering of cartilage. In: Bronzino JD, ed. The Biomedical Engineering Handbook. Boca Raton: CRC Press, 1995:1778-1796.
2. Freed LE, Vunjak G, Biron RJ et al. Biodegradable polymer scaffolds for tissue engineering. Bio/Technology 1994; 20:689-693.
3. Freed LE, Vunjak-Novakovic G, Langer R. Cultivation of cell-polymer cartilage implants in bioreactors. J Cell Biochem 1993; 51:257-264.
4. Freed LE, Vunjak-Novakovic G. Cultivation of cell-polymer constructs in simulated microgravity. Biotech Bioeng 1995; 46:306-313.
5. Vunjak-Novakovic G, Freed LE, Biron RJ et al. Effects of mixing on the composition and morphology of tissue engineered cartilage. AIChE J 1996; 42:850-860.
6. Freed LE, Vunjak-Novakovic G. Bioreactor cultivation of tissue engineered cartilage. 1996 (manuscript in preparation).
7. Freed LE, Marquis JC, Nohria A et al. Neocartilage formation *in vitro* and *in vivo* using cells cultured on synthetic biodegradable polymers. J Biomed Mat Res 1993; 27:11-23.
8. Vacanti C, Langer R, Schloo B et al. Synthetic biodegradable polymers seeded with chondrocytes provide a template for new cartilage formation in vivo. Plast Reconstr Surg 1991; 88:753-759.
9. Freed LE, Grande DA, Lingbin Z et al. Joint resurfacing using allograft chondrocytes and synthetic biodegradable polymer scaffolds. J Biomed Mater Res 1994; 28:891-899.
10. Schwarz RP, Goodwin TJ, Wolf DA. Cell culture for three-dimensional modeling in rotating-wall vessels: an application of simulated microgravity. J Tiss Cult Meth 1992; 14:51-58.
11. Lillie RD, Fullmer HM. Histopathologic techniques and practical histochemistry. 4th ed. New York: McGraw Hill, 1976.
12. Kim YJ, Sah RL, Doong JYH et al. Fluorometric assay of DNA in cartilage explants using Hoechst 33258. Anal Biochem 1988; 174:168-176.
13. Farndale RW, Buttle DJ, Barrett AJ. Improved quantitation and discrimination of sulphated glycosaminoglycans by the use of dimethylmethylene blue. Biochim Biophys Acta 1986; 883:173-177.
14. Woessner JF. The determination of hydroxyproline in tissue and protein samples containing small proportions of this amino acid. Archiv Biochem Biophys 1961; 93:440-447.
15. Levenspiel O. Chemical reaction engineering. 2nd Ed. New York: Wiley and Sons, 1972: 253-314.
16. Croughan MS, Hamel JF, Wang DIC. Hydrodynamic effects on animal cells grown in microcarrier cultures. Biotech Bioeng 1987; 29:130-141.
17. Papoutsakis ET. Fluid mechanical damage of animal cells in bioreactors. Trends Biotech 1991; 9:427-437.
18. Clift R, Grace JR, Weber ME. Bubbles Drops and Particles. New York: Academic Press, 1978: 142-168.
19. Willmarth WW, Hawk NE, Harvey R. Steady and unsteady motions and wakes of freely falling discs. The physics of fluids 1964; 7:197-208.
20. Buckwalter JA, Rosenberg LC, Hunziker EB. Articular cartilage: composition, structure, response to injury, and methods of facilitating repair. In: Ewing JW ed. Articular Cartilage and Knee Function: Basic Science and Arthroscopy. New York: Raven Press, 1990: 19-55.
21. Setton LA, Zhu W, Mow VC. The biphasic poroviscoelastic behavior of articular cartilage: role of the surface zone in governing the compressive behavior. J Biomech 1993; 26:81-592.

22. Bursac, PM, Freed, LE, Biron, RJ et al. Mass transfer studies of tissue engineered cartilage. Tissue Engineering 1996 (accepted with revisions).
23. Dunkelman N, Zimber MP, LeBaron RG et al. Cartilage production by rabbit articular chondrocytes on polyglycolic acid scaffolds in a closed bioreactor system. Biotech Bioeng 1995; 46:299-305.
24. Gurdon JB. A community effect in animal development. Nature 1988; 336:772-774.
25. Tacchetti C, Tavella S, Dozin B et al. Cell condensation in chondrogenic differentiation. Exper Cell Res 1992; 200:26-33.
26. Brittberg M, Lindahl A, Nilsson A et al. Treatment of deep cartilage defects in the knee with autologous chondrocyte transplantation. NEJM 1994; 331:889-895.
27. Nevo Z, Robinson D, Halperin N. The use of grafts composed of cultured cells for repair and regeneration of cartilage and bone. In: Hall BK, ed. Bone: Fracture, Repair and Regeneration. Boca Raton: CRC Press, 1992:123-152.
28. Salter RB. The biological concept of continuous passive motion of synovial joints: the first 18 years of basic research and its clinical application. In: Ewing JW, ed. Articular Cartilage and Knee Function: Basic Science and Athroscopy. New York: Raven Press, 1990: 335-352.
29. Thompson DW. On Growth and Form. New York: Cambridge University Press, 1977: 221-267.
30. Sah RLY, Kim YJ, Doong JYH et al. Biosynthetic response of cartilage explants to dynamic compression. J Orthop Res 1989; 7:619-636.
31. Parkkinen JJ, Ikonen J, Lammi MJ et al. Effects of cyclic hydrostatic pressure on proteoglycan synthesis in cultured chondrocytes and articular cartilage explants. Arch Biochem Biophys 1993; 300:458-465.
32. Prewett, TL, Goodwin, TJ, Spaulding, AF. Three dimensional modeling of T-24 human bladder carcinoma cell line: a new simulated microgravity culture vessel. J Tiss Cult Meth 1993; 15:29-36.
33. Langer R, Vacanti JP. Tissue Engineering. Science 1993; 260:920-926.